

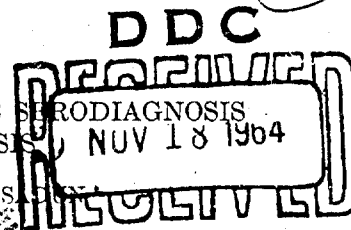
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Printed in U.S.A.⑥ FLUORESCENT ANTIBODY TEST FOR THE SERODIAGNOSIS  
OF VISCERAL LEISHMANIASIS

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An unequivocal diagnosis of visceral leishmaniasis (kala-azar) is often difficult to obtain since the clinical picture is not always well defined and the organisms frequently cannot be readily recovered for microscopic examination. Consequently, the need for reliable and rapid procedures which could provide the basis for an adequate diagnosis of this infection has been evident for a long time. Although agglutinin, precipitin, and complement-fixing antibodies can be demonstrated in animals artificially immunized with cultures of *Leishmania donovani* and to a lesser degree in infected humans, these tests have so far shown only limited value for the routine laboratory diagnosis of this infection.

Following the early unsuccessful attempts to detect specific antibodies in kala-azar infections,<sup>1-4</sup> a complement-fixation test was developed which was regarded as a fairly adequate means of diagnosing human infection.<sup>5, 6</sup> Difficulties were encountered, however, in preparing sufficient quantities of specific antigen for use in the complement-fixation test. Extensive cross-reactions reported with acid-fast bacilli led to the widespread use of these organisms in place of the specific antigen.<sup>6-9</sup> However, as one would expect, when these non-specific antigens were used cross-reactions were observed in lepromatous lepers and in individuals with active tuberculosis. Pellegrino and Brenner performed the complement-fixation test on blood which had been collected on filter paper and allowed to dry at room temperature.<sup>10</sup> They suggested that this method might be used for surveys on canine visceral leishmaniasis.

The growth of *Leishmania* in media containing serum has been reported.<sup>11, 12</sup> Based on these observations, recently Adler was able to detect species and strain specific antibodies by study of the effects of homologous and heterologous sera on the growth of the organisms on semi-solid culture media.<sup>13</sup> Although this may prove to be a very important research tool, its practical applica-

tion in the serological diagnosis of visceral leishmaniasis is doubtful.

Recent investigations<sup>14, 15</sup> resulted in the development of a fluorescent antibody (FA) test for the serodiagnosis of trypanosomiasis in experimental animals and man. Relatively few cross-reactions were observed with sera from healthy controls and from patients with non-trypanosomal diseases, thus indicating a high degree of specificity of this reaction. It was also observed that blood dried on absorbent paper could be tested successfully with this technique,<sup>16</sup> thus providing a useful tool for epidemiologic investigations in endemic areas.

The above results obtained with African and American trypanosomes suggested investigations of the use of the fluorescent antibody method in the diagnosis of visceral leishmaniasis. The current report summarizes results of studies in which the FA technique was used to stain culture forms of *L. donovani* for the purpose of developing a reliable test for the laboratory diagnosis of kala-azar. The procedures were evaluated with sera obtained from humans with proven kala-azar infections in endemic areas. Attempts were made to determine the degree of cross-reactivity with sera from individuals infected with different *Leishmania* species and from those with several other viral, mycotic, and parasitic infections. Furthermore, studies were carried out to determine whether blood smears dried on paper could be used in the serological diagnosis of visceral leishmaniasis.

## MATERIALS AND METHODS

**Sera.** Thirty human sera were obtained from individuals with well-documented visceral leishmaniasis infections. In most of the patients, diagnosis was established by the identification of organisms recovered by spleen biopsy. A total of 196 control sera from individuals with viral, bacterial, and other parasitic infections as well as sera from individuals with degenerative

diseases was used in determining the specificity of the test. Normal control sera were obtained from 22 healthy donors who were undergoing physical examinations as candidates for appointment to the U. S. Military Academy. All sera were heated at 56°C for 30 minutes and diluted 1:16 with 0.01 M phosphate buffered saline (PBS) at pH 7.0 to 7.2 before testing. In addition to serum specimens, dried blood smears were prepared on filter paper from individuals with a proven diagnosis of leishmaniasis. Samples of serum and dried blood from one kala-azar patient were compared as to their suitability for use in the test. The dried blood smears were prepared, extracted, and tested as previously described.<sup>16</sup>

**Test procedure.** The antigen used in the test was the leptomonal form of *Leishmania donovani* (Khartoum strain) cultivated on diphasic blood agar medium. Approximately 0.3 ml of overlay was removed from a culture of *L. donovani* and washed twice in PBS by centrifugation for 5 minutes at 1,000 × G. The resulting concentrate provided material for 50 or more tests. The tests were conducted on microscope slides on each of which was drawn a circle of "decorite" paint (Craftint Mfg. Co., Cleveland, Ohio) approximately 2 cm in diameter.<sup>14</sup> The circle of paint aided in locating the organisms on the slide and in conserving solutions used in the test by limiting them to the area of the circle. A thin smear of concentrated organisms was made within the circle with an applicator stick or wire loop. The smears were allowed to dry for at least 10 minutes, but no further drying was allowed during the subsequent steps of the procedure. The entire technic was carried out at room temperature (22° to 24°C). Fluorescent staining was accomplished by the indirect FA technic<sup>17</sup> using a commercially prepared fluorescein-labeled antihuman globulin. The highest dilution of conjugate giving a bright fluorescence to the *Leishmania* with homologous antiserum and essentially no fluorescence with normal serum was used. With few exceptions, a 1:15 dilution fulfilled these requirements. A 0.002% aqueous solution of Evans blue<sup>18</sup> was used as a counterstain. After comparison was made between the use of rhodamine bovine albumin<sup>19</sup> and Evans blue<sup>18, 19</sup> as a counterstain, the latter was chosen for use in the present test because it provided better contrast between results obtained with reactive and non-reactive sera. Titration of sera with and without the counterstain detected

no inhibition of specific fluorescence by use of this technic. The *Leishmania* smears were covered with 5% formalin and fixed for 10 minutes. The test area was then washed three times with PBS. One-tenth ml of previously heated and diluted test serum was applied with a pipette and allowed to react for 10 minutes. The washing procedure was repeated, and 0.1 ml of previously titrated labeled antihuman globulin was added. After 5 minutes of exposure to the antiglobulin, the test area was again washed three times with PBS and 0.1 ml of Evans blue solution was added and allowed to react for 10 minutes. Following a final rinsing three times in PBS, a drop of buffered glycerol was placed on the slide and surmounted with a 22 x 22 mm cover glass. The slide was blotted gently to remove the excess fluid.

**Visualization.** Fluorescence microscopy was accomplished with a Zeiss photomicroscope with ultra-violet assembly (air-cooled mercury vapor lamp Osram HBO-200) with five excitor filters and eight combinations of barrier filters covering the entire ultraviolet range. The excitor filter BG-12 (4 mm) and barrier filter 50 special yellow gave the best results. The degree of fluorescence seen in the leptomonal forms was estimated according to criteria described previously,<sup>20</sup> and the results were classified as reactive (4+, 3+, 2+), weakly reactive (1+), and non-reactive (± and -). Reactivity was indicated by the presence of a yellow-green fluorescence, non-reactivity by a predominantly red or red-brown color.

#### RESULTS

A marked degree of yellow-green fluorescence was observed on the leptomonal forms where immune sera were used in the indirect FA technic. This contrasted vividly with the light red fluorescence observed with normal sera in the same system.

The relative sensitivity of the technic is indicated by results obtained with sera from visceral leishmaniasis patients (Table 1). Findings based on sera from individuals with other diseases and from healthy controls indicate the degree of specificity of the test. Cross-reactions were obtained with sera from patients with mucocutaneous leishmaniasis. Among individuals with other proven parasitic, viral, and bacterial infections, cross-reactions occurred occasionally with the sera from malaria, American trypanosomiasis, schistosomiasis, and leprosy patients. Twenty-one of 22 healthy control sera were non-

TABLE 1  
Results obtained in the fluorescent antibody test  
for visceral leishmaniasis in human sera

Diagnostic status	No. tested	Reaction	Weak reaction	No reaction
Visceral leishmaniasis	30	21	5	4
<i>Other infections</i>				
Mucocutaneous leishmaniasis	31	7	5	19
Cutaneous leishmaniasis	5	0	1	4
African trypanosomiasis	16	0	1	15
American trypanosomiasis	13	2	0	11
Malaria	23	3	2	18
Amebiasis	2	0	0	2
Trichinosis	8	0	0	8
Strongyloidiasis	5	0	0	5
Onchocerciasis	6	0	1	5
Schistosomiasis	15	1	2	12
Syphilis	12	0	1	11
Leprosy	15	1	1	13
Tuberculosis	10	0	0	10
Systemic mycoses*	27	0	1	26
<i>Degenerative diseases</i>				
Lupus erythematosus	8	1	0	7
Healthy controls	22	0	1	21

\* All 27 were positive in one or more tests for histoplasmosis, blastomycosis or coccidioidomycosis; 5 were from patients with coccidioidomycosis proven by recovery of organisms.

reactive in the test; the remaining serum specimen was weakly reactive.

Comparison between serum samples and dried blood smears from the same kala-azar patient indicated a close correlation between results obtained with the two types of specimens. Tests performed with these dried blood smears after storage at room temperature indicated that positive results could be obtained for at least 2 months following collection. Similar samples from kala-azar patients tested approximately 15 months after collection gave no reaction in the test.

#### DISCUSSION

The results of these experiments, conducted with a total of 248 human sera, reveal that specific antibodies are produced in man following active infection with *Leishmania donovani*. These

antibodies are detectable in a reproducible manner by the indirect fluorescent antibody technic, using as antigen leptomonad forms of *L. donovani*.

Few cross-reactions occurred with sera from individuals infected with trypanosomes. These results are of major interest because of the close phylogenetic relationships between *Leishmania* and *Trypanosoma* and are in agreement with and support the findings observed by Sadun *et al.*<sup>15</sup> in the development and evaluation of a FA test for the serodiagnosis of trypanosomiasis in man. Similarly, only a few cross-reactions were observed with sera from individuals with syphilis, leprosy and tuberculosis. This observation is particularly significant in view of the fact that positive complement-fixation tests for leishmaniasis using *L. donovani* as antigen have been reported in patients who had a positive Wassermann reaction<sup>21, 22</sup> and in those infected with acid-fast organisms.<sup>6</sup>

Since some of the sera from patients with proven infections with *L. donovani* gave a negative reaction, it is quite possible that the FA test described here may be somewhat deficient in sensitivity. This may be due in part to the fact that leptomonad forms were used as a source of antigen and these forms have some antigenic dissimilarities with the *Leishmania* forms in the mammalian host.<sup>23-25</sup> If so, the test might be improved by using leishmanial forms as a source of antigen either from cultures incubated at high temperatures<sup>26</sup> or from impression smears or sections from organs of heavily infected experimental animals.

A few serum samples from patients with proven infections of malaria reacted positively in this test. Although this may be due to the fact that some of these sera were obtained from Africa and Southeast Asia where kala-azar is present, the possibility should be considered that true cross-reactivity exists between *Plasmodia* and *Leishmania*. Adler observed that leishmanial infections with virulent strains of *L. infantum* in hamsters confer marked protection against *Plasmodium berghei* and against *Babesia rodhaini*.<sup>27</sup>

Cross-reactions were also observed in individuals infected with mucocutaneous leishmaniasis. Conversely, an apparent species specificity was observed in this test when sera from patients infected with *L. tropica* were used. This may be simply due to the fact that in man *L. tropica* fails to elicit production of antibodies in

sufficient amounts to be readily detectable by serological tests. This observation was made by Adler who failed to detect agglutinating antibodies in the serum of patients with *L. tropica*.<sup>28</sup> To collect additional information on this important question, sera from two patients with proven *L. tropica* infections were also tested in the FA reaction using *L. tropica* as antigen. These sera failed to react with the homologous as well as with the heterologous antigen, thus adding to the already existing evidence of poor antibody production in man following active infection with *L. tropica*.

As observed in schistosomiasis,<sup>16, 29</sup> trichinosis,<sup>30</sup> and trypanosomiasis,<sup>15</sup> the FA technic permitted reliable testing for *L. donovani* fluorescent antibodies even when dried blood smears were used instead of serum. The ability to utilize minute amounts of dried blood, which can be easily obtained and mailed to a central laboratory, suggests that after appropriate standardization this test may be used to advantage in epidemiologic investigations of visceral leishmaniasis. However, sufficient information is not yet available regarding the sensitivity and specificity of the FA test and its applicability to field conditions to justify its recommendation for the routine laboratory diagnosis of kala-azar.

#### SUMMARY

The indirect fluorescein-labeled antibody technic was used and evaluated in the serodiagnosis of kala-azar in humans. The tests were performed on slides using *Leishmania donovani* leptomonad forms as antigen. Evans blue, employed as a counter-stain, resulted in improved contrast, thus making the test easier to interpret, yet did not significantly diminish the specific yellow-green fluorescence.

Occasional cross-reactions were observed with specimens from individuals with viral, bacterial, mycotic, and parasitic infections as well as with degenerative diseases. Although cross-reactions were frequently observed in individuals with mucocutaneous leishmaniasis, negative results were reported from *L. tropica* patients. Reliable testing for visceral leishmaniasis fluorescent antibodies was possible even when dried blood smears were used instead of serum.

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